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APPLICATION NUMBER: ***60/558,080***
FILING DATE: ***March 30, 2004***

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

H. L. JACKSON
Certifying Officer

Practitioner's Docket No. 61149 P (49434) ***PATENT***

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Alan H. LAZARUS, Vinayakumar SIRAGAM, Davor BRINC, John FREEDMAN, Seng SONG, and Andrew R. CROW.

For:

METHOD FOR TREATING THROMBOCYTOPENIA WITH ANTIBODIES

Mail Stop Provisional Patent Application
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

COVER SHEET FOR FILING PROVISIONAL APPLICATION (37 C.F.R. § 1.51(c)(1))

WARNING: *"A provisional application must also include the cover sheet required by § 1.51(c)(1) or a cover letter identifying the application as a provisional application. Otherwise, the application will be treated as an application filed under paragraph (b) [nonprovisional application] of this section." 37 C.F.R. § 1.53(c)(1). See also M.P.E.P. § 201.04(b), 6th ed,. rev. 3.*

NOTE: "A complete provisional application does not require claims since no examination on the merits will be given to a

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CERTIFICATION UNDER 37 C.F.R. § 1.10*
*(Express Mail label number is **mandatory**.)*
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I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on **March 30, 2004** , in an envelope as Express Mail Label No. **EV438992624US** addressed to Mail Stop Provisional Patent Application, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

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provisional application. However, provisional applications may be filed with one or more claims as part of the application. Nevertheless, no additional claim fee or multiple dependent claims fee will be required in a provisional application." Notice of December 5, 1994, 59 Fed. Reg. 63,951, at 63,953.

"Any claim filed with a provisional application will, of course, be considered part of the original provisional application disclosure." Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,209.

NOTE: "A provisional application is not entitled to the right of priority under 35 U.S.C. 119 or 365(a) or § 1.55, or to the benefit of an earlier filing date under 35 U.S.C. 120, 121 or 365(c) or § 1.78 of any other application. No claim for priority under § 1.78(a)(3) may be made in a design application based on a provisional application. No request under § 1.293 for a statutory invention registration may be filed in a provisional application. The requirements of §§ 1.821 through 1.825 regarding application disclosures containing nucleotide and/or amino acid sequences are not mandatory for provisional applications." 37 C.F.R. § 1.53(c)(3).

NOTE: "No information disclosure statement may be filed in a provisional application." 37 C.F.R. § 1.51(d). "Any information disclosure statements filed in a provisional application would either be returned or disposed of at the convenience of the Office." Notice of December 5, 1994, 59 Fed. Reg. 63,591, at 63,594.

NOTE: "No amendment other than to make the provisional application comply with the patent statute and all applicable regulations, may be made to the provisional application after the filing date of the provisional application. " 37 C.F.R. § 1.53(c).

***WARNING:** A provisional application may be abandoned by operation of 35 U.S.C. 111(b)(5) on a Saturday, Sunday, or Federal holiday within the District of Columbia, in which case, a nonprovisional application claiming benefit of the provisional application under 35 U.S.C. 119(e) must be filed no later than the preceding day that is not a Saturday, Sunday, or Federal holiday within the District of Columbia. Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,202.*

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. § 1.51(c)(1)(i).

1. The following comprises the information required by 37 C.F.R. § 1.51(c)(1):

2. The name(s) of the inventor(s) is/are (37 C.F.R. § 1.51(c)(1)(ii)):

NOTE: "If the correct inventor or inventors are not named on filing, a provisional application without a cover sheet under § 1.51(c)(1), the later submission of a cover sheet under § 1.51(c)(1) during the pendency of the application will act to correct the earlier identification of inventorship." 37 C.F.R. § 1.48(f)(2).

NOTE: "The naming of inventors for obtaining a filing date for a provisional application is the same as for other applications. A provisional application filed with the inventors identified as 'Jones et al.' will not be accorded a filing date earlier than the date upon which the name of each inventor is supplied unless a petition with the fee set forth in § 1.17(i) is filed which sets forth the reasons the delay in supplying the names should be excused. Administrative oversight is an acceptable reason. It should be noted that for a 35 U.S.C. 111(a) application to be entitled to claim the benefit of the filing date of a provisional application the 35 U.S.C. 111(a)[,] application must have at least one inventor in common with the provisional application." Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,209.

The term "invention" is typically used to refer to subject matter which applicant is claiming in his/her application. Because claims are not required in a provisional application, it would not be appropriate to reference joint inventors as those who have made a contribution to the "invention" disclosed in the provisional application. If the "invention" has not been determined in the provisional application because no claims have been presented, then the name(s) of those person(s) who have made a contribution to the subject matter disclosed in the provisional application should be submitted. Section 1.45(c) states that "if multiple inventors are named in a provisional application, each named inventor must have made a contribution, individually or jointly, to the subject matter disclosed in the provisional application." All that § 1.45(c) requires is that if someone is named as an inventor, that person must have made a contribution to the subject matter disclosed in the provisional application. When applicant has determined what the invention is by the filing of the 35 U.S.C.

111(a) application, that is the time when the correct inventors must be named. The 35 U.S.C. 111(a) application must have an inventor in common with the provisional application in order for the 35 U.S.C. 111(a) application to be entitled to claim the benefit of the provisional application under 35 U.S.C. 119(e). Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,208.

See 37 C.F.R. § 1.53.

1.	Alan **GIVEN NAME**	H **MIDDLE INITIAL OR NAME**	LAZARUS **FAMILY (OR LAST) NAME**
2.	Vinayakumar **GIVEN NAME**	 **MIDDLE INITIAL OR NAME**	SIRAGAM **FAMILY (OR LAST) NAME**
3.	Davor **GIVEN NAME**	 **MIDDLE INITIAL OR NAME**	BRINC **FAMILY (OR LAST) NAME**
4.	John **GIVEN NAME**	 **MIDDLE INITIAL OR NAME**	FREEDMAN **FAMILY (OR LAST) NAME**
5.	Seng **GIVEN NAME**	 **MIDDLE INITIAL OR NAME**	SONG **FAMILY (OR LAST) NAME**
6.	Andrew **GIVEN NAME**	R **MIDDLE INITIAL OR NAME**	CROW **FAMILY (OR LAST) NAME**

4. The title of the invention is (37 C.F.R. § 1.51(c)(1)(iv)):

METHOD FOR TREATING THROMBOCYTOPENIA WITH ANTIBODIES

5. The name, registration, customer and telephone numbers of the practitioner *(if applicable)* are (37 C.F.R. § 1.51(c)(1)(v)):

Name of practitioner: Dianne M. Rees, Ph.D.

Reg. No. 45,281 Tel. (617) 439-4444

Customer No. 21,874

(complete the following, if applicable)

[ ] A power of attorney accompanies this cover sheet.

6. The docket number used to identify this application is (37 C.F.R. § 1.51(c)(1)(vi)):

Docket No. 61149 P (49434)

7. The correspondence address for this application is (37 C.F.R. § 1.51(c)(1)(vii)):

EDWARDS & ANGELL, LLP, P.O. BOX 55874, Boston, Massachusetts 02205

8. Statement as to whether invention was made by an agency of the U.S. Government or under contract with an agency of the U.S. Government. (37 C.F.R. § 1.51(c)(1)(viii)).

This invention was made by an agency of the United States Government, or under contract with an agency of the United States Government.

[X] No
[ ] Yes

The name of the U.S. Government agency and the Government Grant Number are:

9. Identification of documents accompanying this cover sheet:

A. Documents required by 37 C.F.R. §§ 1.51(c)(2)-(3):

Specification: No. of pages **20**
Drawings: (FIGS. 1 – 5c) No. of sheets **9**

B. Additional documents:

[X] Claims: No. of claims **11**

Note: See 37 C.F.R. § 1.51.

[ ] Power of attorney
[ ] Small entity statement
[ ] Assignment
[X]Other **(Abstract)**

NOTE: Provisional applications may be filed in a language other than English as set forth in existing § 1.52(d). However, an English language translation is necessary for security screening purposes. Therefore, the PTO will require the English language translation and payment of the fee mandated in § 1.52(d) in the provisional application. Failure to timely submit the translation in response to a PTO requirement will result in the abandonment of the provisional application. If a 35 U.S.C. 111(a) application is filed without providing the English language translation in the provisional application, the English language translation will be required to be supplied in every 35 U.S.C. 111(a) application claiming priority of the non-English language provisional application. Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,209.

10. Fee

The filing fee for this provisional application, as set in 37 C.F.R. § 1.16(k), is $160.00, for other than a

small entity, and $80.00, for a small entity.

[ ] Applicant is a small entity.
[X] Applicant is not a small entity.

NOTE: "A . . . statement in compliance with existing § 1.27 is required to be filed in each provisional application in which it is desired to pay reduced fees." Notice of April 14, 1995, 60 Fed. Reg. 20,195, at 20,197.

11. Small entity statement

[ ] Applicant's state that this is a filing by a small entity under 37 C.F.R. §§ 1.9 and 1.27.

12. Fee payment

[X] Fee payment in the amount of **$ 160.00**

[ ] No filing fee is to be paid at this time. (This and the surcharge required by 37 C.F.R. § 1.16(l) can be paid subsequently.)

13. Method of fee payment

[X] Check in the amount of **$ 160.00**
[ ] Charge Account No. _____, in the amount of $ _____.
A duplicate of this Cover Sheet is attached.

Please charge Account No. 04-1105 for any fee deficiency.

Date: March 30, 2004

Dianne Rees

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Attorney Docket No. 61149 P (49434)

METHOD FOR TREATING THROMBOCYTOPENIA WITH ANTIBODIES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This is the first application filed for the present invention.

TECHNICAL FIELD

[0002] This application relates to the treatment of thrombocytopenia using antibodies.

BACKGROUND OF THE INVENTION

[0003] Immune thrombocytopenic purpura (ITP) is an autoimmune disease characterised by platelet clearance mediated by pathogenic anti-platelet antibodies. It is thought that this platelet clearance is mediated by Fcγ receptor (FcγR)-bearing macrophages in the reticuloendothelial system (RES). While intravenous immunoglobulin (IVIg) is widely used in the treatment of ITP as well as in a wide variety of chronic autoimmune and inflammatory diseases, its mechanism of action is not yet fully elucidated. Possible mechanisms of action include inhibition of RES function, anti-idiotype antibodies and immunomodulation. In murine models of ITP, it has been demonstrated that IVIg ameliorates ITP by a mechanism completely dependent upon the expression of the inhibitory FcγRIIB. In humans, there is also evidence that IVIg increases the level of expression of FcγRIIB. In addition, the clinical effects of IVIg as well as monoclonal mimetics of IVIg both ameliorate murine ITP in a manner that correlates with RES blockade; this 'competitive' RES

blockade has long been considered to be the primary mechanism whereby IVIg ameliorates ITP.

[0004] The present study was undertaken to investigate if antibodies to soluble antigens could inhibit or reverse ITP. In particular, IgG targeted to a soluble vs. a cell-bound antigen was compared in the effectiveness of treating murine ITP.

SUMMARY OF THE INVENTION

[0005] In one embodiment of the invention there is provided a method for treating thrombocytopenia in a mammal which method comprises administering to the mammal an effective amount of at least one antibody specific for a soluble antigen for a time and under conditions sufficient to inhibit platelet clearance. The soluble antigen can either be an endogenous or a foreign antigen. By foreign antigen it is meant an antigen that is not normally produced by the same individual or species.

[0006] In an aspect of the invention the soluble foreign antigen and the antibody can be incubated together to form antibody-antigen complexes prior to administering the complexes to the mammal.

[0007] In another aspect of the invention, the endogenous soluble antigen can be obtained from the mammal and incubated with the antibody to form antibody-antigen complexes, the complexes being subsequently administered to the mammal.

[0008] The antibody can be administered intravenously, interperitoneally, intramuscularly ,subcutaneously, orally or rectally.

[0009] In another embodiment of the invention, the soluble antigen can be associated with blood cells and the resulting antigen-cell complexes can be targeted by antibodies for inhibiting platelet clearance and thereby treating thrombocytopenia.

[0010] In another aspect of the invention there is provided pharmaceutical compositions for treating thrombocytopenia, comprising an effective amount of at least one antibody specific for a soluble antigen and/or for a soluble antigen associated with a blood cell.

[0011] In yet another aspect of the invention, we demonstrate herein that antibodies to soluble antigens can ameliorate ITP in an FcγRIIB-dependent manner. Antibody directed to the cell-associated antigen inhibited ITP in an FcγRIIB-independent manner. Taken together, these data demonstrate that IgG antibodies reactive with either a soluble or insoluble antigen can mimic the effects of IVIg. In addition, the mechanisms of action of these moieties are quite different: antibody reacted with soluble antigen may utilize the same pathway used by IVIg, i.e. an FcγRIIB-dependent pathway, whereas antibody reacted with a cell-associated antigen may work by another mechanism altogether, possibly by competitive RES inhibition.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] Further features and advantages of the present invention will become apparent from the following detailed description, taken in combination with the appended drawings, in which:

[0013] Figs. 1A and 1B illustrate the association of OVA on the surface of RBCs wherein OVA coupled RBCs are prepared with 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDAC) (Sigma Oakville, ON).OVA was coupled to RBCs as follows: RBCs were resuspended at 2.5x108/mL in 5 mg/mL OVA in saline and 1.9 mg/mL EDAC was added. Following a 1 hr incubation at 4oC, the cells were washed once with a 2 mg/mL solution of OVA in phosphate buffered saline (PBS), pH 7.2 followed by one wash in PBS. The OVA coupled RBCs were stained with rabbit (Fig. 1A) or mouse (Fig. 1B) polyclonal anti-OVA IgG (solid histogram), control rabbit (Fig. 1A) or mouse (Fig. 1B) IgG (solid line), followed by the appropriate FITC conjugated secondary antibody (dashed line, secondary antibody only) and wherein the *x* axis shows relative fluorescence intensity; *y*-axis represents cell number;

[0014] Figs. 2A and 2B illustrates inhibition of thrombocytopenia by treating OVA-coupled RBCs with OVA-specific IgG; CD1 mice were pre-injected intravenously with $1x10^8$ OVA-coupled RBCs pre-incubated with either rabbit (A) or mouse (B) OVA-specific polyclonal IgG, control IgG, or anti-OVA antibody, as indicated on the x axis. Mice in the IVIG groups received 50 mg IVIG. All mice (except 'Normal') received anti-platelet antibody one day later. Mice were

bled for platelet enumeration after a further 24 h. Normal: The dashed line denotes the mean platelet count of non-injected mice; ITP: The horizontal bar denotes the mean platelet count (± 1 SEM) of mice injected with anti-platelet antibody only. The x-axis indicates treatment; y-axis denotes platelet count; n=9 mice for each data point. *** $P < 0.001$ vs. ITP mice. Data are represented as mean ±.SEM;

[0015] Figs. 3A, 3B and 3C illustrates amelioration of thrombocytopenia with antibodies reactive with soluble OVA (in combination with soluble OVA) ameliorate immune thrombocytopenia; CD1 mice were pre-injected intravenously with 1 mg OVA pre-incubated with the dose of rabbit polyclonal anti-OVA (A), or mouse monoclonal anti-OVA (B), as indicated on the x axis. Mice in the IVIG groups received 50 mg IVIG. The induction of thrombocytopenia and platelet counting were as in figure 2. Panel C: the OVA/polyclonal anti-OVA solution was centrifuged and the supernatant fluid filtered using a 0.2 um filter to remove macromolecular immune complexes. The pellet was resuspended in PBS. Mice were injected with the therapeutic preparations indicated on the x axis. The induction of thrombocytopenia, platelet counting, and axis legends are as in Fig 2. The number of mice for data point were n=15 (A, B), n=4 (C). *** $P < 0.001$ vs. ITP mice. Data are represented as mean ± SEM;

[0016] Figs. 4A and 4B illustrates inhibition of RES function by antibodies reactive with soluble OVA (Fig. 4A) or OVA-RBCs (Fig. 4B). Panel A: Mice were either not pre-

treated (O), pre-treated with IVIG (□), pre-treated with 1 mg OVA pre-incubated with 1 mg rabbit anti-OVA (Δ), or pre-treated with 1 mg control IgG + 1 mg OVA (▼), followed 24 hours later by intravenous injection with fluorescently labeled TER-119-opsonized syngeneic RBCs, prepared as follows: Whole blood (2 ml, diluted with 1/5 volume 1% EDTA in PBS) from unmanipulated mice was pooled and centrifuged at 2,000 x g for 15 min to obtain 1 ml of packed erythrocytes. These packed erythrocytes were resuspended in 4 ml of PBS and incubated with 8 μg of anti-TER-119 antibody at 22°C for 30 min. The resulting opsonized erythrocytes were then washed twice with PBS and labeled with a fluorescent marker (PKH26 Kit, Sigma, St. Louis MO) as follows: Briefly, the opsonized erythrocytes were resuspended in 3 ml of PKH26 'diluent C' and mixed with another 4 ml of 'diluent C' containing 10 μl of the 'PKH26 linker'. After a 5 minute incubation with constant swirling, the mixture was incubated for 5 minutes with an equal volume of PBS containing 1% bovine serum albumin. The erythrocytes were washed 5 times and resuspended in 2 ml PBS. Mice were then injected via the tail vein with 200 μl of these labeled cells. All mice were bled via the tail vein at 3 min, 10 min, 30 min, 120 min, and 960 min post injection and the total number of erythrocytes, as well as the percent of PKH26-fluorescent erythrocytes, were counted by flow cytometry. The percentage of labeled erythrocytes at the 3 min time point was considered to be 100%.

[0017] Blood samples were taken at the times indicated on the x axis and the percentage of fluorescent RBCs in the circulation assessed by flow cytometry (Fig. 4B), mice were

either not pre-treated (O), pre-treated with IVIG (□), pre-treated with anti-OVA sensitized OVA-RBCs (Δ), or pre-treated with OVA-RBCs only (▼) followed 24 hours later with intravenous injection of fluorescently labeled TER-119-opsonized autologous RBCs;

[0018] Fig. 5 illustrates that antibodies reactive with soluble OVA or OVA-RBCs both ameliorate immune thrombocytopenia independent of complement activity wherein mice were injected with CVF to deplete complement or were left untreated and after 24 hours, mice were treated with the therapeutic preparations indicated on the x axis, the induction of thrombocytopenia and platelet counting were as in Fig 2, control: mice receiving no therapeutic pre-treatment; Nil: mice treated with anti-platelet antibody only; 'OVA + anti-OVA': mice pre-treated with OVA + anti-OVA, followed 24 hr later by injection of anti-platelet antibody. 'OVA-RBC + anti-OVA': mice pre-treated with OVA-RBC + anti-OVA, followed 24 hr later by injection of anti-platelet antibody;

[0019] Figs. 6A and 6B illustrate that FcγRIIB expression is required for reversal of immune thrombocytopenia by soluble OVA in the presence of anti-OVA wherein wild type mice (Fig. 16A) or mice genetically deficient for FcγRIIB (FcγRIIB-/-) mice (Fig. 6B) were injected with anti-platelet antibody on days 0 through 3 denoted by the arrow (↑), on day 2 (↓) mice were injected intraperitoneally with IVIG (□), or intravenously with OVA + anti-OVA antibody (Δ), or non-specific IgG + OVA (▼) and mice were bled daily for platelet counts;

[0020] Figs. 7A and 7B illustrate that FcγRIIB expression is not required for reversal of immune thrombocytopenia by cell-associated OVA in the presence of anti-OVA wherein wild type mice (Fig. 7A) or FcγRIIB$^{-/-}$ mice (Fig. 7B) were injected with anti-platelet antibody on days 0 through 3 (↑), on day 2 (↓) mice were injected intraperitoneally with IVIG (□), or intravenously with anti-OVA sensitized OVA-RBCs (△), or OVA-RBCs alone; and

[0021] Figs. 8A and 8B illustrate that antibodies to endogenous soluble antigens ameliorate immune thrombocytopenia wherein (Fig. 8A) mice were treated with IVIG only (□), 10 mg OVA (△), or 1 mg OVA (O), followed four hours later by 1 mg OVA-specific IgG (↓) on day 2 and wherein thrombocytopenia and platelet counting were as in Fig 6 and wherein (Fig. 8B) mice were treated with IVIG (□), 1 mg anti-mouse albumin antibody (▲), 1 mg anti-mouse transferrin antibody (O), or control IgG (♦).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

[0022] In this description by soluble antigen it is meant a molecule that can be incorporated and circulated in the blood stream. Examples of soluble antigens comprise but are not limited to: proteins, glycoproteins, lipids, glycolipids, peptides, nucleic acids, synthetic molecules or complexes or aggregates thereof.

[0023] By endogenous antigen it is meant antigens that occur naturally in a mammal and by foreign (or exogenous)

antigen it is meant an antigen that is not normally produced by the same individual or species.

[0024] Antibodies to soluble antigens were tested for their ability to ameliorate thrombocytopenia. To address this question, a murine model of ITP was used to determine whether IgG specific to a soluble prototype antigen could prevent thrombocytopenia. Mice injected with soluble ovalbumin (OVA) or OVA conjugated to RBCs (OVA-RBC) in the presence of anti-OVA, were both significantly protected from immune thrombocytopenia.

[0025] Both of these therapeutic regimes functioned independent of complement activity and both regimes also blocked reticuloendothelial function as assessed by clearance rates of fluorescent sensitized syngeneic RBCs. Soluble OVA or anti-OVA alone did not have any direct effect on immune thrombocytopenia in mice. It was found that OVA-RBC + anti-OVA ameliorated immune thrombocytopenia in $Fc\gamma RIIB^{-/-}$ mice, while soluble OVA + anti-OVA was ineffective. In addition, IgG specific for murine albumin and specific for transferrin also effectively inhibited ITP. Thus IgG antibodies directed to soluble antigens can inhibit or reverse immune thrombocytopenia in an FcγRIIB-dependent manner, whereas antibodies directed to a cell-associated antigen function independent of FcγRIIB expression.

Materials and Methods

Reagents

[0026] The monoclonal antibody specific for integrin α_{IIb} (rat $IgG_{1\kappa}$, clone MWReg 30) was purchased from BD Pharmigen

(Mississauga, ON, Canada). Monoclonal murine anti-OVA (IgG_1, clone OVA-14), rabbit polyclonal anti-OVA, 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDAC), OVA (grade V), and PKH26 red fluorescent cell linker kit were purchased from Sigma (Oakville, ON, Canada). IVIG was Gamimune,10% from Bayer (Elkhart, IN). Cobra Venom Factor (CVF), FITC-conjugated $F(ab')_2$ anti-rabbit IgG, and control rabbit IgG, were purchased from Cedarlane Laboratories Ltd (Hornby, ON, Canada). Rabbit anti-mouse albumin (IgG fraction), and rabbit anti-mouse transferrin (IgG fraction), were purchased from Research Diagnostics (Flanders, NJ). Hemolysin (anti-SRBC rabbit serum) was supplied by Colorado Serum company (Denver, CO). Microdispenser tubes (250 µl) for blood collection were from VWR, (Mississauga, ON)

Mice

[0027] Female CD1 mice (6-8 wks of age) were purchased from Charles River Laboratories (Montreal, PQ, Canada). C57BL/6 and FcγRIIB$^{-/-}$ (B6;129S4-*Fcgr2b*^{tm1Rav}/J) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All mice were housed in the St. Michael's Hospital Research Vivarium.

Induction and treatment of immune thrombocytopenia

[0028] Mice were rendered thrombocytopenic by intraperitoneal injection of 2 µg anti-platelet (anti-integrin α_{IIb}) antibody in 200 µl phosphate buffered saline (PBS), pH 7.2. ITP was induced by two protocols:

[0029] For experiments where the therapeutic intervention preceded the induction of immune thrombocytopenia (e.g.

Figs 2, 3, 5), mice were first injected intravenously with the indicated therapeutic preparation (eg OVA-RBC sensitized with anti-OVA IgG), followed at 24 h by a single injection of anti-platelet antibody. Mice were bled for platelet enumeration after a further 24 h.

[0030] For experiments where the induction of immune thrombocytopenia preceded the therapeutic intervention (e.g. Figs 6-8), mice were injected daily (days 0-3) with anti-platelet antibody and then injected intravenously with the indicated therapeutic preparation (eg OVA-RBC sensitized with anti-OVA IgG) on day 2. Mice were bled daily and platelets counted as described below.

[0031] In experiments where we wished to avoid the possibility of the formation of "pre-formed" immune complexes, mice were injected intraperitoneally with soluble OVA only followed 4 hours later by OVA-specific antibody via the intravenous route. Mice injected with anti-albumin or anti-transferrin alone received 1 mg of antibody in a volume of 200 ul on day 2. For all IVIg treatments, mice were injected intraperitoneally with 0.5 ml of 10 % IVIG (roughly equivalent to 2 g/kg body weight). Platelets were counted as follows: Mouse blood (45 μL) was collected via saphenous vein bleeding into microdispenser tubes preloaded with 5 µl of 1% EDTA in PBS. Then, 50 µl of this mouse blood was diluted in 450 µl of 1% EDTA/PBS (1:10) and then further diluted to a final dilution of 1:12,000 in 1% ethylenediaminetetraacetic acid (EDTA)/PBS. Platelets were enumerated on a flow rate-calibrated FACScan flow cytometer (Becton Dickinson, San Jose, CA) using

forward scatter (FCS) versus side scatter (SSC) to gate platelets as previously described.

Preparation of OVA-specific antibody pre-incubated with soluble OVA

[0032] 1 mg OVA was dissolved in 300 µl PBS and was incubated with the indicated dose (Fig. 3A, 3B x-axes) of OVA-specific antibody (rabbit polyclonal or mouse monoclonal) for 1 hr at 37°C. The solution was then injected intravenously in a 300µl volume. In separate experiments the OVA and antibody solution was incubated as above for 1 hour at 37°C and macromolecular immune complexes removed by centrifugation at 16,000xg at 4°C for 1 h followed by filtration of the resulting supernatant fluid using a 0.2 µm filter (Filtropur S plus 0.2, Sarstedt, Montreal, PQ). The pellet was resuspended in 300 µl PBS and intravenously injected as above.

Preparation of OVA-coupled RBCs

[0033] OVA was coupled to RBCs as follows: RBCs were resuspended at $2.5x10^8$/mL in 5 mg/mL OVA in saline and 1.9 mg/mL 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDAC) was added. Following a 1 hr incubation at 4°C, the cells were washed once with a 2 mg/mL solution of OVA in PBS followed by one wash in PBS. To confirm the presence of OVA on RBCs, OVA coupled RBCs were incubated with 17 µg/mL rabbit polyclonal anti-OVA, washed, and then incubated with 8 µg/mL FITC conjugated $F(ab')_2$ anti-rabbit IgG. Cells were washed, resuspended in PBS, and analyzed by flow cytometry.

Reticuloendothelial system (RES) blockade

[0034] RES blockade was assessed as follows: Whole blood (2 ml, diluted with 1/5 volume 1% EDTA in PBS) from unmanipulated SCID mice was pooled and centrifuged at 2,000 x g for 15 min to obtain 1 ml of packed erythrocytes. These packed erythrocytes were resuspended in 4 ml of PBS and incubated with 8 μg of anti-TER-119 antibody at 22^{o}C for 30 min. The resulting opsonized erythrocytes were then washed twice with PBS and labeled with a fluorescent marker (PKH26 Kit, Sigma, St. Louis MO) according to the manufacturer's directions. Briefly, the opsonized erythrocytes were resuspended in 3 ml of PKH26 'diluent C' and mixed with another 4 ml of 'diluent C' containing 10 μl of the 'PKH26 linker'. After a 5 minute incubation with constant swirling, the mixture was incubated for 5 minutes with an equal volume of PBS containing 1% bovine serum albumin. The erythrocytes were washed 5 times and resuspended in 2 ml PBS. Mice were then injected via the tail vein with 200 μl of these labeled cells. All mice were bled via the tail vein at 3 min, 10 min, 30 min, 120 min, and 960 min post injection and the the total number of erythrocytes, as well as the percent of PKH26-fluorescent erythrocytes, were counted by flow cytometry. The percentage of labeled erythrocytes at the 3 min time point was considered to be 100%.

Complement depletion

[0035] Complement depleted mice were prepared by intraperitoneal injection of 5U of Cobra Venom Factor (CVF) in 200 µl phosphate-buffered saline pH 7.2 followed by a second injection of CVF after 4h. Complement depletion was

confirmed by the complement hemolytic activity assay Briefly, sheep RBCs (SRBC) were washed in PBS and resuspended at $1x10^8$/mL. Hemolysin (anti-SRBC rabbit serum) was diluted 1:50 and incubated with these sheep RBCs at 37°C for 30 min, washed in PBS and the cells incubated with a 1:10 dilution of mouse sera from control vs. CVF-treated mice at 37°C for 30 min. The mixture was then diluted with PBS, centrifuged at 1000 xg for 5 min. Complement activity from the sera was assessed as follows: SRBC were resuspended in PBS at 1 x 10^8/mL. One mL of this was incubated with 1 mL of a 1/50 dilution of anti-SRBC antibody ('Hemolysin', Colorado serum, Denver, CO) and incubated for 30 min at 37°C. Cells were washed in PBS, and adjusted to 1 x 10^8/mL in PBS. Twenty mL of these cells were added to 20 µl mouse serum from experimental mice in a 96 well flat bottom tissue culture plate for 30 min at 37°C. The plate was then centrifuged at 1,000 x g for 5 min, the supernatant was transferred to a new 96 well plate and the absorbance was read at 540 nm. Calculate percent hemolysis: 100 x (OD_{540}sample-OD_{540}blank)/(OD_{540}max-OD_{540}blank). Calculate 50% lysis by plotting the log of serum dilution against log (%lysis/(100-%lysis)).

Statistical analysis

[0036] Data was analyzed using the Student's *t* test, except data in Fig. 8, which was analyzed by one-way ANOVA. The level of significance was set at $P< 0.05$.

Results

Antibodies reactive with a cell-associated antigen can inhibit immune thrombocytopenia

[0037] OVA-coupled murine RBCs (OVA-RBC) were assessed for reactivity with mouse (Fig. 1A) and rabbit (Fig. 1B) antibody specific to OVA by flow cytometry to ensure successful coupling of the OVA-RBCs. The monoclonal anti-OVA antibody employed in this study did react with OVA (as assessed by ELISA), but did not react with OVA-RBCs suggesting that the epitope recognized on OVA may be masked upon coupling with RBCs. Thus monoclonal anti-OVA was only used in treatments involving soluble OVA.

[0038] CD1 mice were injected intravenously with $1x10^8$ OVA-RBCs pre-incubated with nothing, OVA specific antibodies or an appropriate control IgG, 50 mg IVIg (roughly equivalent to 2g/kg body weightin a 25g mouse), or were left untreated. After 24 hours, all mice received anti-platelet antibody and all mice were bled for platelet enumeration after a further 24 h. Mice that received anti-platelet antibody alone became thrombocytopenic (Figure 2, shaded horizontal bar), compared to unmanipulated control mice (Figure 2, dashed line). Mice treated with OVA-RBCs pre-incubated with either 50 μg rabbit polyclonal anti-OVA (Figure 2A, 'OVA-RBC + anti-OVA') or 50 μg murine polyclonal anti-OVA (Figure 2B, 'OVA-RBC + anti-OVA') were significantly protected from the development of immune thrombocytopenia compared with mice receiving OVA-RBCs alone (OVA-RBC) or OVA-RBC + control IgG (OVA-RBC + control IgG). The effectiveness of the IgG coated OVA-RBCs was comparable to that of IVIg (Figure 2A&B).

Antibodies reactive with a soluble antigen can inhibit immune thrombocytopenia

[0039] CD1 mice were injected intravenously with 1 mg soluble OVA that had been pre-incubated with serial dilutions of the indicated amount of rabbit polyclonal anti-OVA (Figure 3A) or the indicated amount of murine monoclonal anti-OVA antibody (Figure 3B) one day prior to injection of anti-platelet antibody. Both of these therapeutic preparations ameliorated immune thrombocytopenia (polyclonal anti-OVA at dosages of 1.0 or 0.5 mg/mouse, monoclonal at dosages of 50 or 10 ug/mouse). It is of interest to note that OVA incubated with 50 ug monoclonal anti-OVA was essentially as successful at inhibiting ITP as was a standard dose of IVIg (Fig 3B). Mice treated with soluble OVA alone (Figure 3A&B, 0.0 mg/mouse) or OVA + control IgG (data not shown) were not significantly protected from the development of immune thrombocytopenia. OVA by itself did not affect the platelet count at any dose tested (0.1 mg, 1 mg, 5 mg and 10 mg, data not shown). Similarly all of the anti-OVA antibodies, in the absence of OVA, did not inhibit immune thrombocytopenia (data not shown).

[0040] To determine if the OVA + anti-OVA preparation ameliorated immune thrombocytopenia due to the formation of large macromolecular immune complexes, we subjected the OVA + polyclonal anti-OVA preparation (1mg:1mg) to centrifugation at 16,800 xg for 1 hr. at 4°C and the resulting supernatant was then filtered through a 0.2 uM filter (Filtropur S plus 0.2, Sarstedt, Montreal, PQ). Pretreatment of mice with the filtered supernatant, but not the dissolved pellet(the pellet was dissolved by resuspending the pellet in PBS, pH 7.2, back to the

original volume), prior to injection of anti-platelet antibody protected mice from thrombocytopenia (Figure 3C), suggesting that the "active" fraction was soluble and less than 0.2 uM in size.

Antibodies reactive with soluble and a cell-associated soluble antigen both block RES function

[0041] To assess whether the therapeutic regimes under study inhibited RES function, we employed a variation of the classic RES blockade assay, analysing the clearance rate of fluorescently labelled syngeneic RBCs sensitised with a murine RBC-specific antibody (anti-TER-119). Mice were subjected to the indicated therapeutic treatments, and their ability to clear these intravenously injected labelled RBCs over time was analysed (Fig 4). For the soluble antigen studies, mice were injected with nothing, IVIg, OVA-anti-OVA, or control IgG alone for 24 h followed by sensitized fluorescent RBCs (Figure 4A). At the indicated times post sensitized-fluorescent-RBC injection, blood was sampled to assess the RBC clearance rate as a measure of RES function. Only IVIg and OVA-anti-OVA blocked sensitized RBC clearance. Similar results were obtained with murine anti-OVA in combination with soluble OVA (data not shown).

[0042] For the cell-associated antigen studies, mice were injected with nothing, IVIg, anti-OVA sensitized OVA-RBCs, or OVA-RBCs alone for 24 h followed by sensitized fluorescent RBCs (Figure 4B). Only IVIg and anti-OVA sensitized OVA-RBCs blocked sensitized-fluorescent-RBC clearance.

Antibodies reactive with soluble or cell-associated soluble antigen inhibit ITP independent of complement activity

[0043] To determine if complement was a contributing factor to the above therapies, mice were depleted of Complement using cobra factor venom (CVF) as described above in [46]. CVF successfully depleted complement from the treated mice as assessed in a hemolytic activity assay on day 3 post CVF-treatment (data not shown). Complement depleted mice developed thrombocytopenia to the same extent as normal mice (Figure 5, column set 2). Complement depleted and normal mice both responded to the protective effects of OVA + anti-OVA and OVA-RBC + anti-OVA (column sets 4 and 5 respectively) to the same extent. However, complement depleted mice responded to IVIg treatment with significantly higher platelet counts compared with normal mice.

FcγRIIB expression is required for protection with antibodies reactive with soluble, but not a cell-associated antigen.

[0044] Wild type and FcγRIIB$^{-/-}$ mice were injected daily with anti-platelet antibody (↑) to induce stable thrombocytopenia (Fig 6). Mice were then treated with IVIg, OVA + anti-OVA, or control IgG + OVA on day 2. Treatment of mice with 2 g/kg IVIg as well as OVA + anti-OVA successfully reversed immune thrombocytopenia in wild type (Figure 6A), but neither ameliorated ITP in FcγRIIB$^{-/-}$ mice (Figure 6B). Mice treated with control IgG + OVA displayed no increase in platelet counts. In sharp contrast to the results in Fig 6, ITP was successfully reversed in normal mice (Figure 7A) and FcγRIIB$^{-/-}$ mice (Figure 7B) that were therapeutically treated with OVA-RBCs + anti-OVA. As

expected, treatment of mice with OVA-RBCs alone did not increase platelet counts in thrombocytopenic mice.

Preformation of immune complexes are not necessary for reversal of ITP

[0045] To determine if it is necessary to incubate antigen and antibody before injection to ameliorate the thrombocytopenia in our model, mice were pre-injected with either 1 mg or 10 mg of soluble OVA followed by 1 mg anti-OVA after 4h. Significant reversal of ITP was achieved with OVA specific IgG in mice that were previously treated with either 1mg or 10 mg of OVA (Figure 8A).

[0046] To determine if antibody to endogenous soluble antigens can also inhibit immune thrombocytopenia, thrombocytopenic mice were treated with 1 mg polyclonal anti-mouse albumin or 1 mg anti-mouse transferrin antibody on day 2. Both of these antibodies, but not control IgG, significantly ameliorated the immune thrombocytopenia (Figure 8B). In contrast, anti-mouse albumin and anti-mouse transferrin antibodies failed therapeutically in FcγRIIB$^{-/-}$ mice, and did not reverse immune thrombocytopenia (data not shown).

[0047] The above described antibodies and antibody-antigen and antibody-antigen-cell complexes can be incorporated in pharmaceutical compositions to be injected in the mammal. Such compositions may also comprise a pharmaceutically acceptable carrier as would be known in the art.

[0048] The compositions can be injected in the mammal by several routes of administration comprising intravenously,

interperitoneally, intramuscularly , subcutaneously, orally or rectally.

[0049] It will be appreciated by persons skilled in the art that other antigens and antibodies could also be used according to the above described method to achieve similar results. It will also be appreciated that the method and composition could be applied to mammals, other than mice and rabbits, such as humans.

[0050] The embodiment(s) of the invention described above is(are) intended to be exemplary only. The scope of the invention is therefore intended to be limited solely by the scope of the appended claims.

I/WE CLAIM:

1. A method for treating thrombocytopenia in a mammal which method comprises administering to said mammal an effective amount of at least one antibody specific for a soluble antigen for a time and under conditions sufficient to inhibit platelet clearance.

2. The method as claimed in claim 1 wherein said soluble antigen is a foreign antigen.

3. The method as claimed in claim 2 wherein said soluble foreign antigen is administered to said mammal prior to administering said antibody.

4. The method as claimed in claim 2 wherein said soluble foreign antigen and said antibody are incubated together to form antibody-antigen complexes prior to administering said complexes to said mammal.

5. The method as claimed in claim 3 or 4 wherein said foreign antigen is ovalbumin.

6. The method as claimed in claim 5 wherein said antibody is monoclonal or polyclonal.

7. The method as claimed in claim 1 wherein said soluble antigen is endogenous.

8. The method as claimed in claim 7 wherein said endogenous soluble antigen is obtained from said

mammal and incubated with said antibody to form antibody-antigen complexes, said complexes being administered to said mammal.

9. The method as claimed in claim 7 wherein said soluble endogenous antigen is selected from albumin and transferrin or a combination thereof.

10. The method as claimed in claim 9 wherein said antibody is a polyclonal antibody or monoclonal antibody.

11. The method as claimed in claim 1 wherein said mammal is a human or a non-human mammal.

12. The method according to claim 1, wherein said at least one antibody is administered intravenously, interperitoneally, intramuscularly , subcutaneously, orally or rectally.

13. A method for treating thrombocytopenia in a mammal which method comprises administering to said mammal an effective amount of at least one antibody specific for a soluble antigen associated with a blood cell for a time and under conditions sufficient to inhibit platelet clearance.

14. The method as claimed in claim 13 wherein said soluble antigen is associated with said blood cell outside said mammal and subsequently administered to said mammal prior to administering said antibody.

15. The method as claimed in claim 14 wherein a plurality of blood cells comprising said soluble antigen are incubated with said antibody to form antibody-antigen-blood cell complexes prior to administering said complexes in said mammal.

16. The method as claimed in claim 15 wherein said blood cell is a red blood cell.

17. The method as claimed in claim 16 wherein said antibody is polyclonal.

18. The method as claimed in claim 13 wherein said mammal is a human or a non-human mammal.

19. The method as claimed in claim 13 wherein said soluble antigen is a foreign antigen.

20. The method according to claim 13, wherein said at least one antibody is administered. intravenously, interperitoneally, intramuscularly , subcutaneously, orally or rectally.

21. A method for treating thrombocytopenia in a mammal said method comprising administering to said mammal an effective amount of at least one antibody specific for a soluble antigen for a time and under conditions sufficient to inhibit Reticuloendothelial system (RES) function and to inhibit platelet clearance.

22. The method as claimed in claim 21 wherein said soluble antigen is a foreign antigen.

23. The method as claimed in claim 22 wherein said soluble foreign antigen is administered to said mammal prior to administering said antibody.

24. The method as claimed in claim 22 wherein said soluble foreign antigen and said antibody are incubated together to form antibody-antigen complexes prior to administering said complexes to said mammal.

25. The method as claimed in claim 23 or 24 wherein said foreign antigen is ovalbumin.

26. The method as claimed in claim 25 wherein said antibody is monoclonal or polyclonal.

27. The method as claimed in claim 21 wherein said soluble antigen is endogenous.

28. The method as claimed in claim 27 wherein said endogenous soluble antigen is obtained from said mammal and incubated with said antibody to form antibody-antigen complexes, said complexes being administered to said mammal.

29. The method as claimed in claim 27 wherein said soluble endogenous antigen is selected from albumin and transferrin or a combination thereof.

30. The method as claimed in claim 29 wherein said antibody is polyclonal or monoclonal.

31. The method as claimed in claim 21 wherein said mammal is a human or a non-human mammal.

32. The method according to claim 21, wherein said at least one antibody is administered intravenously, interperitoneally, intramuscularly, subcutaneously, orally or rectally.

33. A method for treating thrombocytopenia in a mammal which method comprises administering to said mammal an effective amount of at least one antibody specific for a soluble antigen associated with a blood cell for a time and under conditions sufficient to inhibit Reticuloendothelial system (RES) function and to inhibit platelet clearance.

34. The method as claimed in claim 33 wherein said non blood cell antigen is associated with said blood cell outside said mammal and subsequently administered to said mammal prior to administering said antibody.

35. The method as claimed in claim 34 wherein a plurality of blood cells comprising said non-blood cell antigen are incubated with said antibody to form antibody-antigen-blood cell complexes prior to administering said complexes in said mammal.

36. The method as claimed in claim 35 wherein said blood cell is a red blood cell.

37. The method as claimed in claim 36 wherein said antibody is polyclonal or monoclonal.

38. The method as claimed in claim 33 wherein said mammal is a human or a non-human mammal.

39. The method as claimed in claim 33 wherein said soluble antigen is a foreign antigen.

40. The method according to claim 33, wherein said at least one antibody is administered intravenously, interperitoneally, intramuscularly,subcutaneously, orally or rectally.

41. A method of inhibiting platelet clearance in a patient in need thereof which comprises administering to the patient a therapeutic composition comprising a therapeutic amount of at least one antibody specific for a soluble antigen and/or for a soluble antigen associated with a blood cell and a pharmaceutically acceptable carrier, said therapeutic amount being sufficient to inhibit platelet clearance in said patient.

42. The method of claim 41, wherein the therapeutic amount of the at least one antibody specific for a soluble antigen and/or for a soluble antigen associated with a blood cell administered ranges from

about 0.1μg to about 1g per kg of body weight per day.

43. The method of claim 42, wherein the at least one antibody specific for a soluble antigen and/or for a soluble antigen associated with a blood cell is administered for a time sufficient to therapeutically increase and maintain platelet cell counts.

44. The method as claimed in claim 41 wherein said soluble antigen is a foreign antigen.

45. The method as claimed in claim 44 wherein said soluble foreign antigen is administered to said mammal prior to administering said antibody.

46. The method as claimed in claim 44 wherein said soluble foreign antigenor said soluble associated with a blood cell and said antibody are incubated together to form antibody-antigen or antibody-antigen-blood cell complexes prior to administering said complexes to said mammal.

47. The method as claimed in claim 44 wherein said foreign antigen is ovalbumin.

48. The method as claimed in claim 47 wherein said antibody is monoclonal or polyclonal.

49. The method as claimed in claim 41 wherein said soluble antigen is endogenous.

50. The method as claimed in claim 49 wherein said soluble endogenous antigen is selected from albumin and transferrin or a combination thereof.

51. The method as claimed in 49 wherein said endogenous soluble antigen is obtained from said mammal and incubated with said antibody to form antibody-antigen complexes, said complexes being administered to said mammal.

52. The method as claimed in claim 41 wherein said mammal is a human or a non-human mammal.

53. The method according to claim 41, wherein said at least one antibody is administered intravenously, interperitoneally, intramuscularly, subcutaneously, orally or rectally..

54. The method as claimed in claim 41 wherein said soluble antigen is associated with said blood cell outside said mammal and subsequently administered to said mammal prior to administering said antibody.

55. The method as claimed in claim 54 wherein a plurality of blood cells comprising said soluble antigen are incubated with said antibody to form antibody-antigen-blood cell complexes prior to administering said complexes in said mammal.

56. The method as claimed in claim 54 wherein said blood cell is a red blood cell.

57. The method as claimed in claim 54 wherein said antibody is polyclonal.

58. The method of claim 41, wherein said antibody is capable of inhibiting Reticuloendothelial System (RES) function.

59. A pharmaceutical composition for treating thrombocytopenia, comprising an effective amount of at least one antibody specific for a soluble antigen and/or for a soluble antigen associated with a blood cell in combination with a pharmaceutically acceptable carrier.

60. The composition as claimed in claim 59, wherein said antibody is capable of inhibiting Reticuloendothelial System (RES) function.

61. The composition as claimed in claim 59 wherein said soluble antigen is a foreign antigen.

62. The composition as claimed in claim 61 wherein said soluble foreign antigen is administered to said mammal prior to administering said antibody.

63. The composition as claimed in claim 61 wherein said soluble foreign antigen or said soluble antigen associated with a blood cell and said antibody are incubated together to form antibody-antigen or antibody-antigen-blood cell complexes prior to administering said complexes to said mammal.

64. The composition as claimed in claim 61 wherein said foreign antigen is ovalbumin.

65. The composition as claimed in claim 64 wherein said antibody is monoclonal or polyclonal.

66. The composition as claimed in claim 59 wherein said soluble antigen is endogenous.

67. The composition as claimed in claim 66 wherein said soluble endogenous antigen is selected from albumin and transferrin or a combination thereof.

68. The composition as claimed in 66 wherein said endogenous soluble antigen is obtained from said mammal and incubated with said antibody to form antibody-antigen complexes, said complexes being administered to said mammal.

69. The composition as claimed in claim 59 wherein said mammal is a human or a non-human mammal.

70. The composition according to claim 59, wherein said at least one antibody is administered intravenously, interperitoneally, intramuscularly , subcutaneously, orally or rectally..

71. The composition as claimed in claim 59 wherein said soluble antigen is associated with said blood cell outside said mammal and subsequently administered to said mammal prior to administering said antibody.

72. The composition as claimed in claim 71 wherein a plurality of blood cells comprising said soluble antigen are incubated with said antibody to form antibody-antigen-blood cell complexes prior to administering said complexes in said mammal.

73. The composition as claimed in claim 71 wherein said blood cell is a red blood cell.

74. The composition as claimed in claim 71 wherein said antibody is polyclonal.

75. The composition as claimed in claim 59, wherein said antibody is capable of inhibiting Reticuloendothelial System (RES) function.

METHOD FOR TREATING THROMBOCYTOPENIA WITH ANTIBODIES

Abstract

The invention provides a method for treating thrombocytopenia in a mammal which comprises administering an effective amount of at least one antibody specific for a soluble antigen (e.g., an antigen associated with a blood cell, such as albumen or transferrin) for a time and under conditions sufficient to inhibit platelet clearance. The invention also provides a pharmaceutical composition for treating thrombocytopenia, comprising an effective amount of at least one antibody specific for a soluble antigen and/or for a soluble antigen associated with a blood cell in combination with a pharmaceutically acceptable carrier.

A

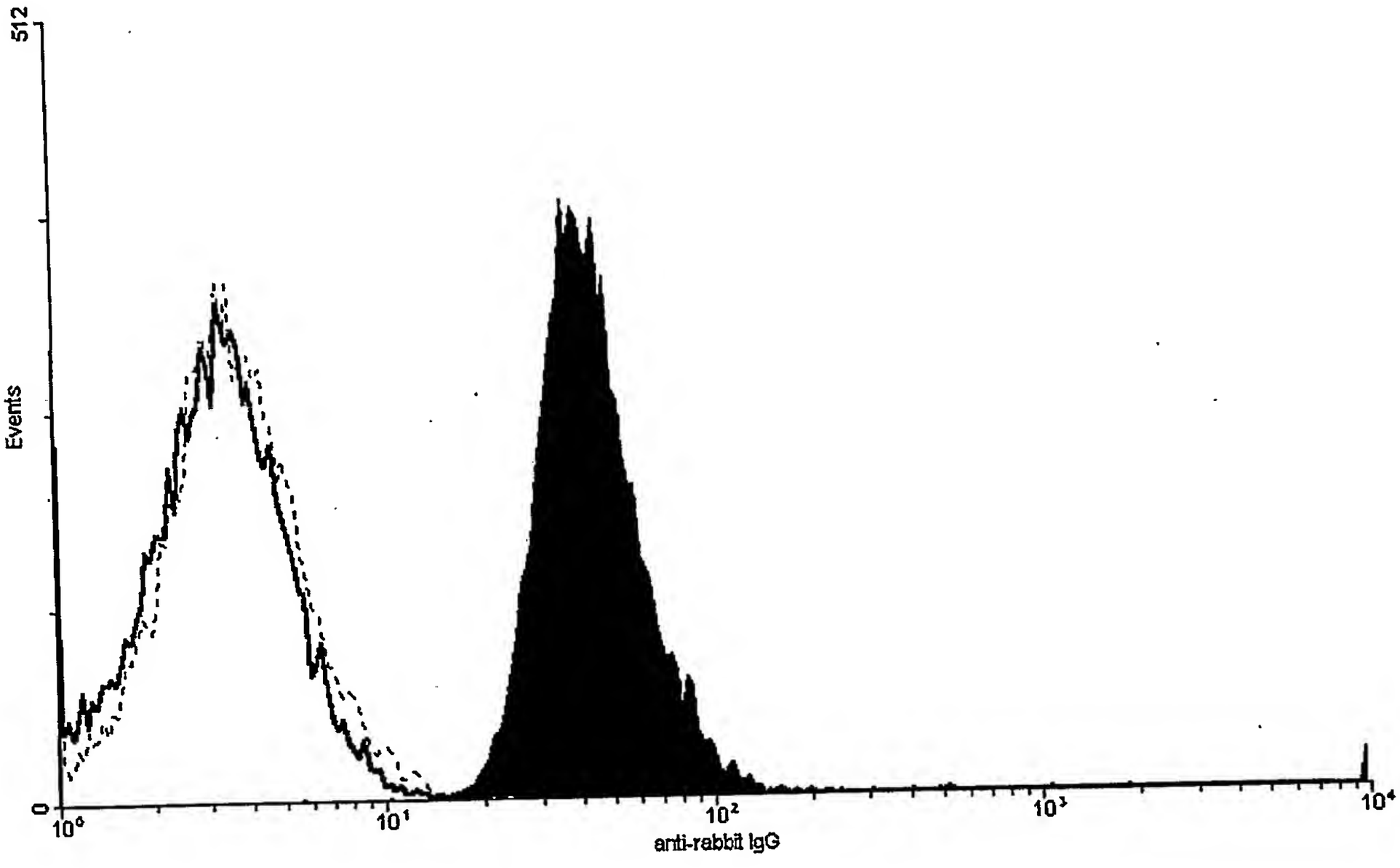

Figure 1A

B

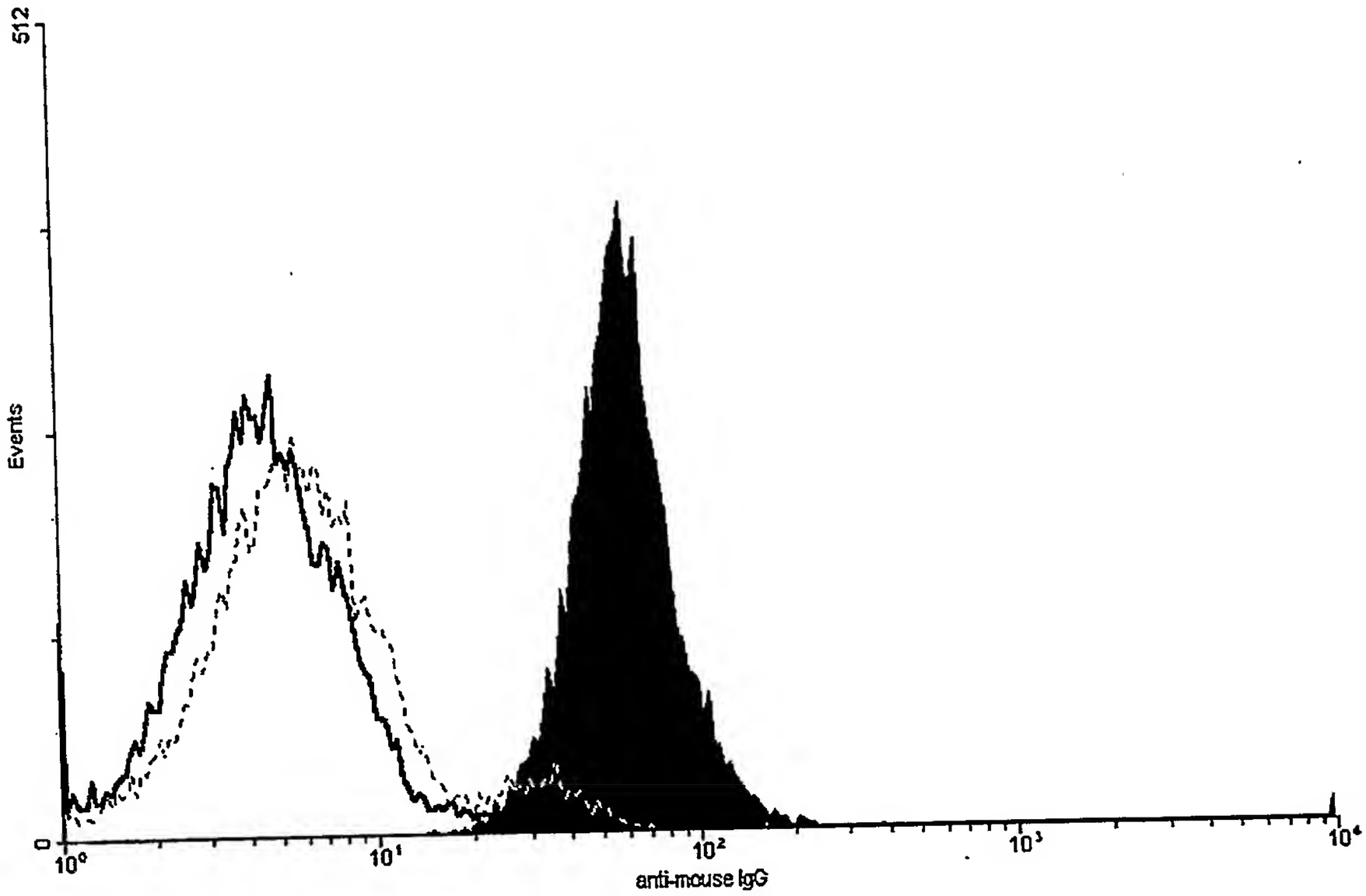

Figure 1B

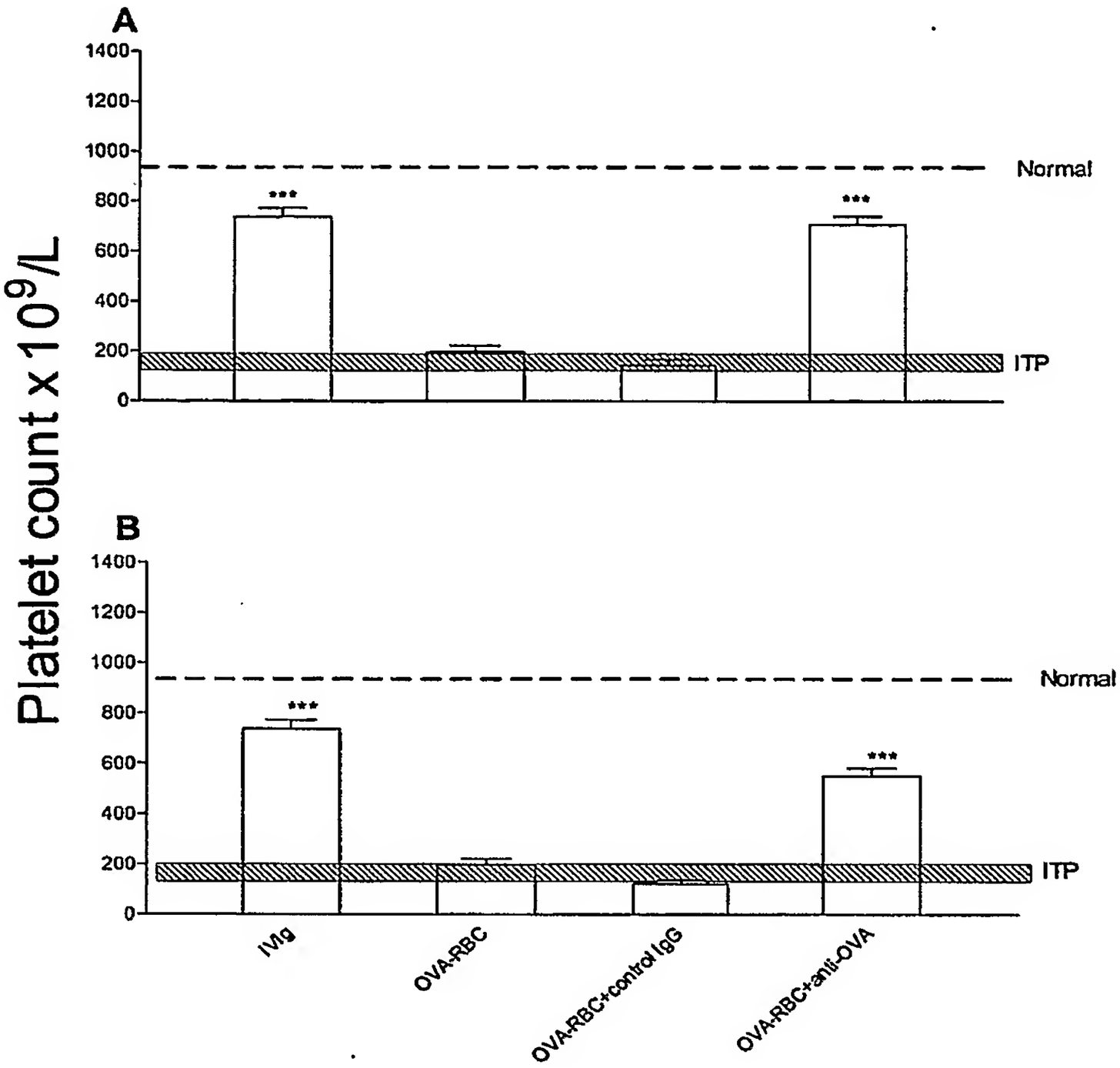

Figure 2

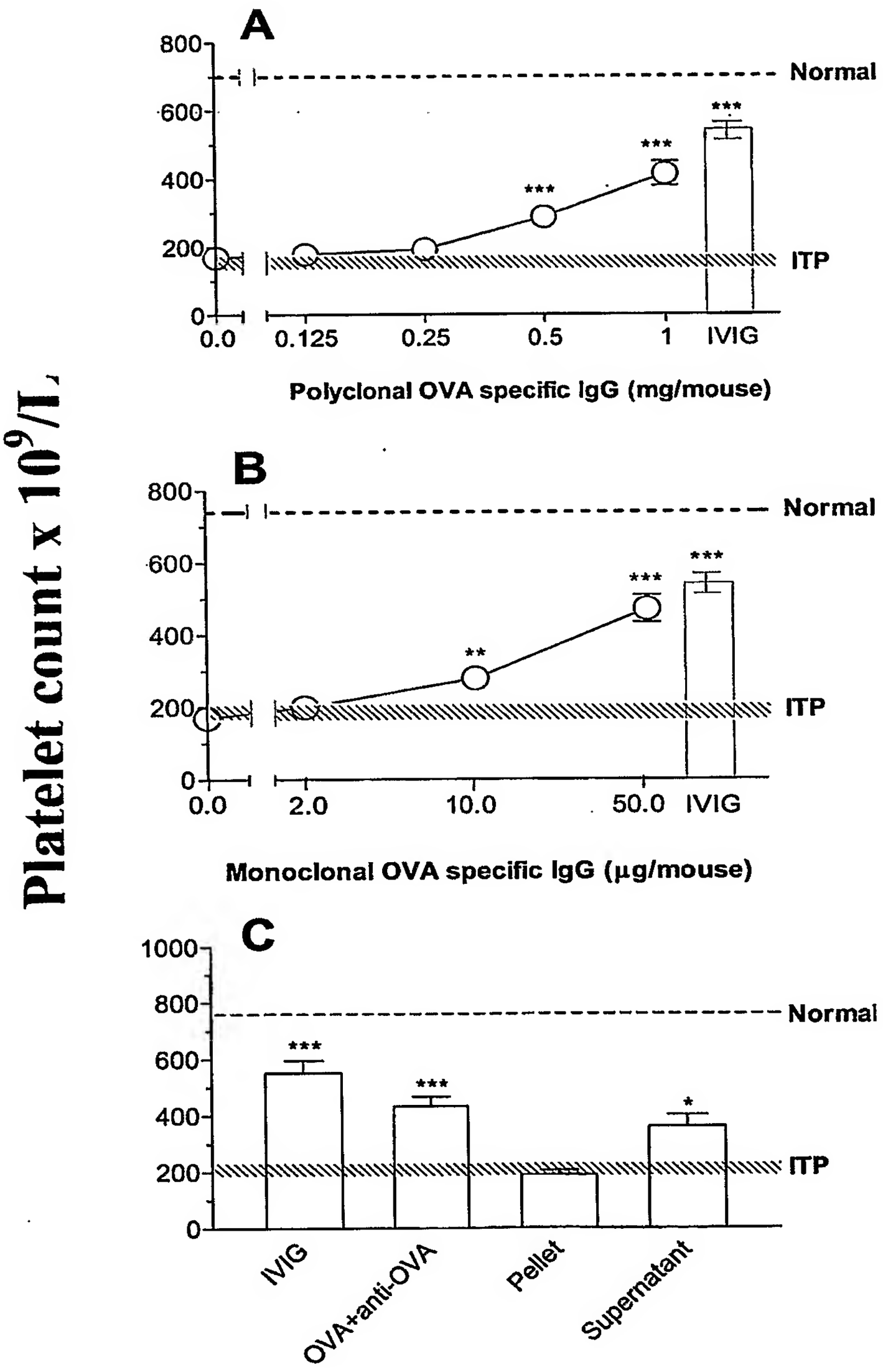

Figure 3

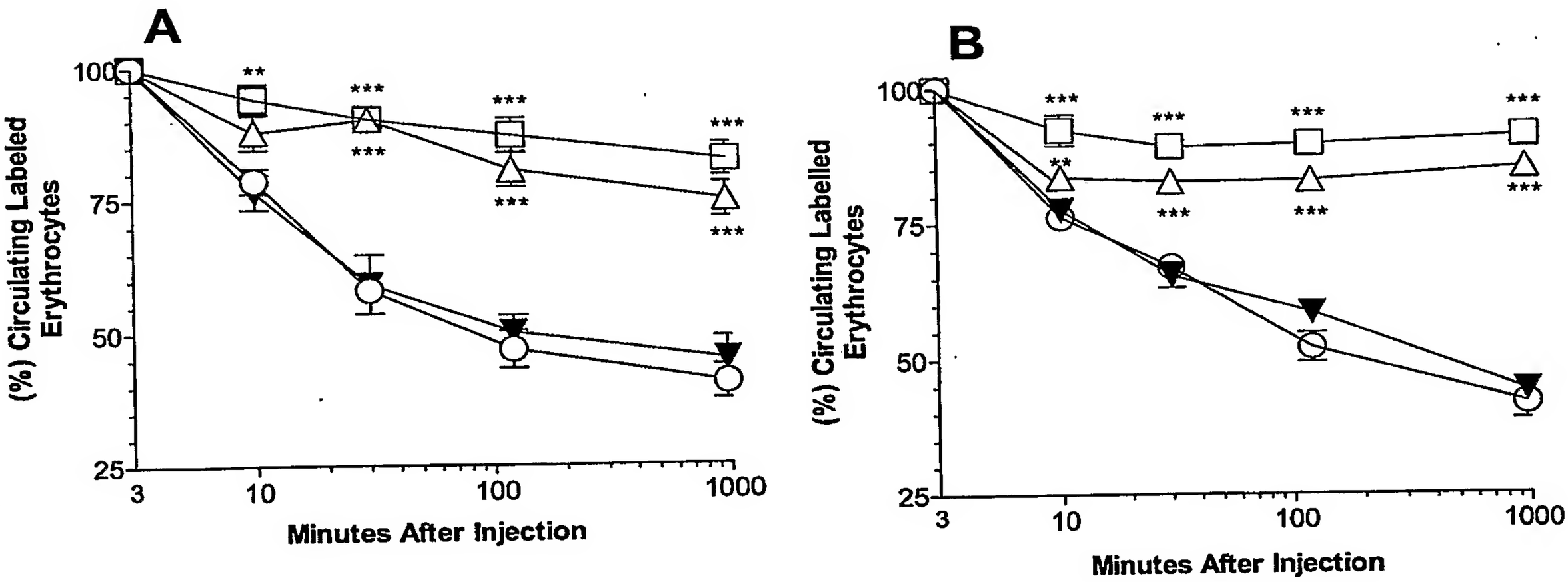

Figure 4

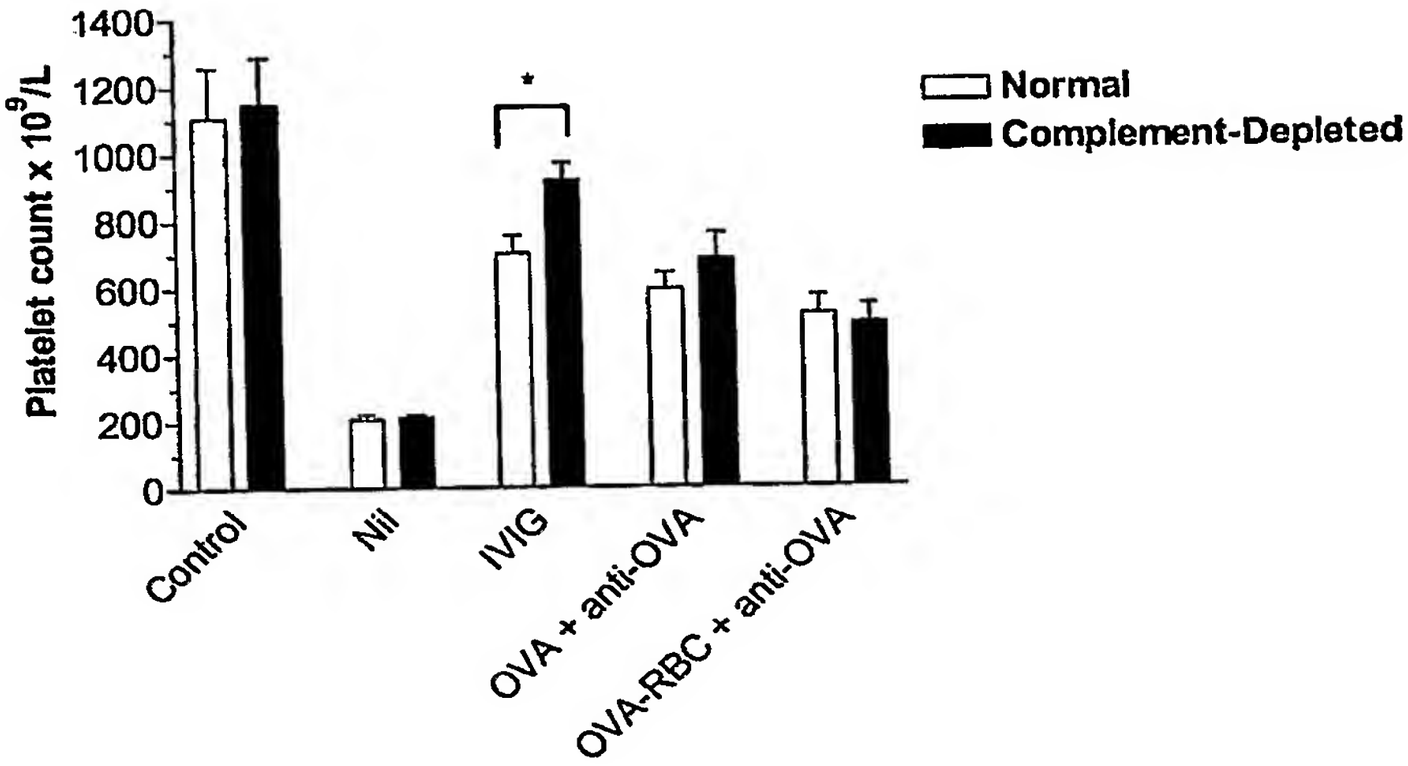

Figure 5

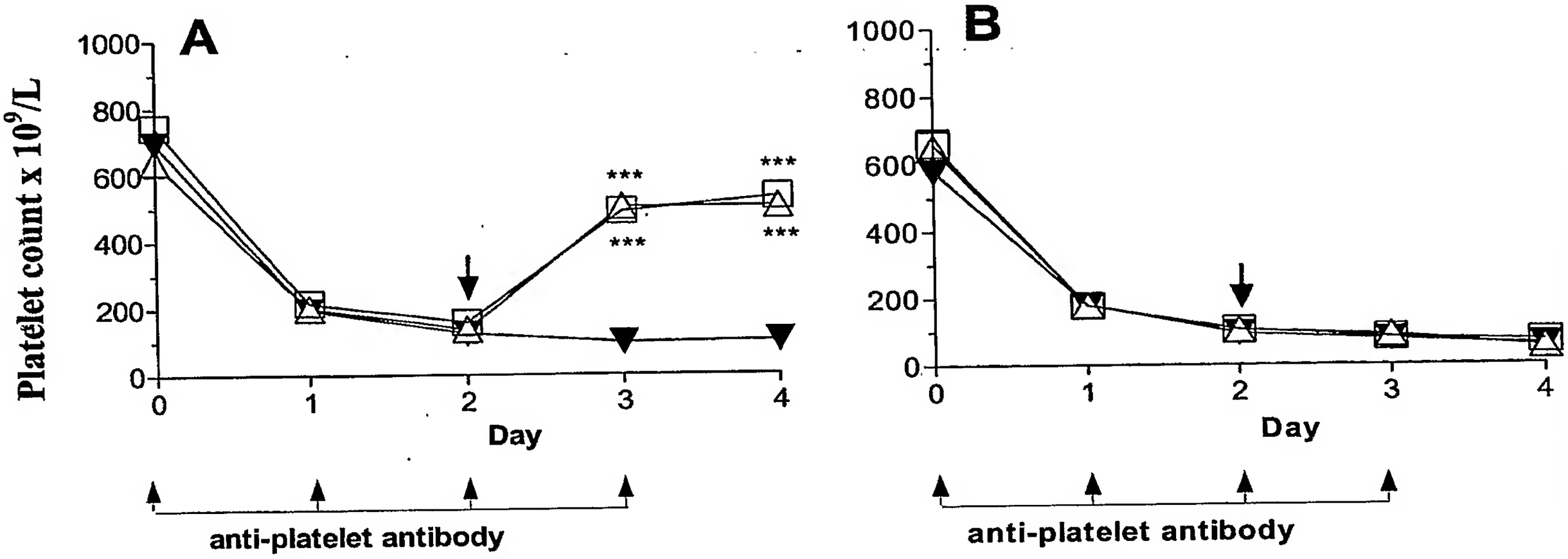

Figure 6

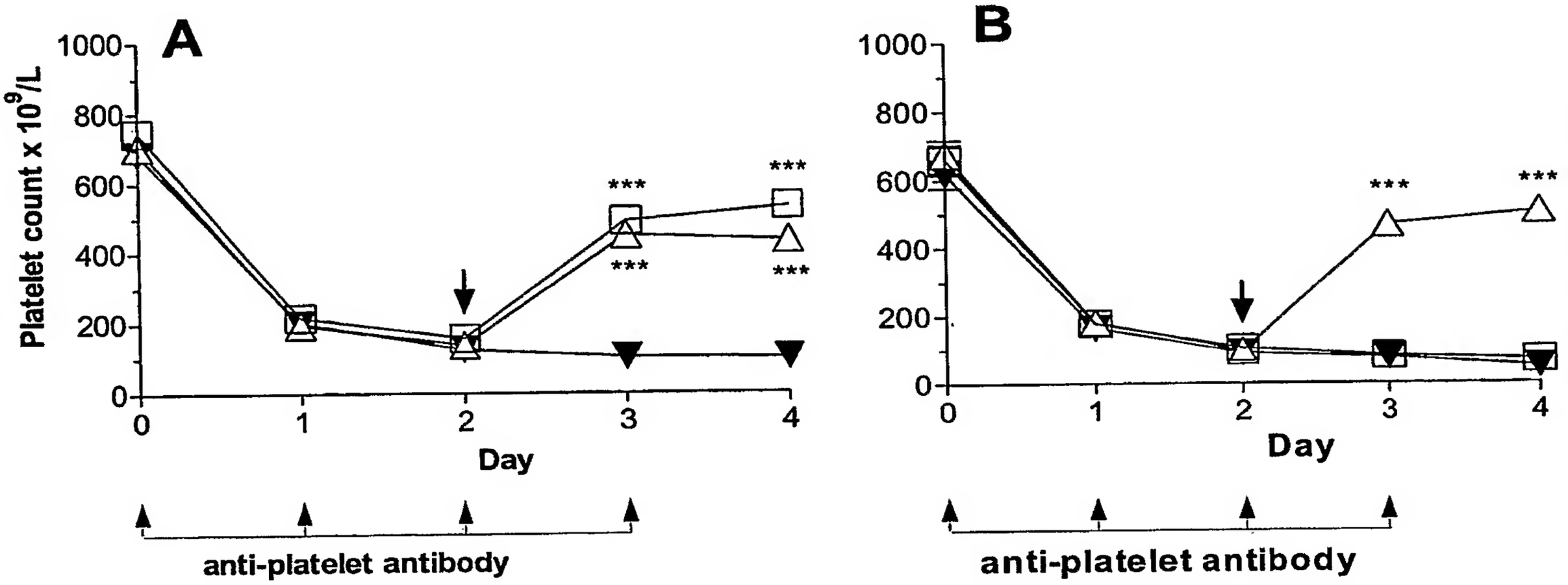

Figure 7

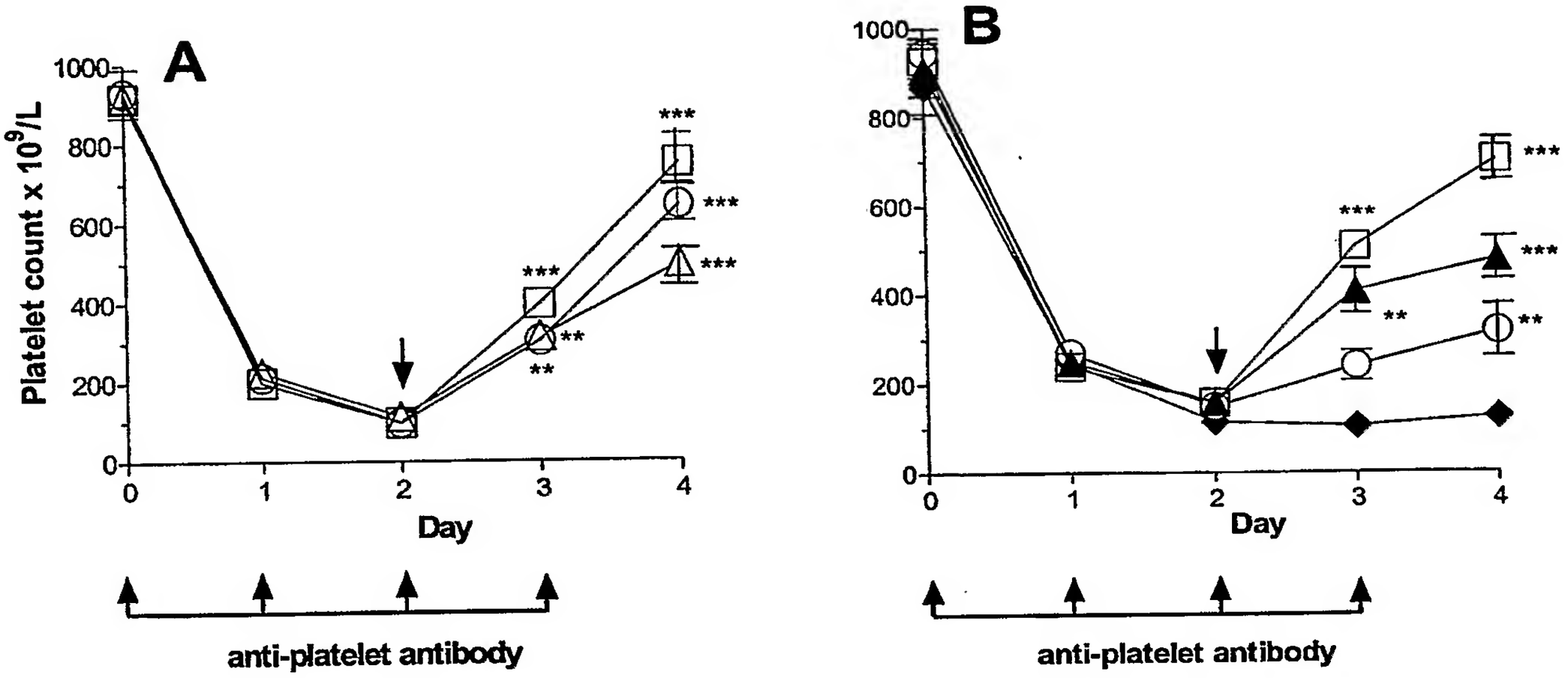

Figure 8